D5 cont.

21. A mutant of a *Escherichia coli* dnaQ49 strain established by the method according to claim 15 or 16, which grows in the presence of 26,000 μg/ml of streptomycin.

22. A mutant of a *Escherichia coli* dnaQ49 strain established by the method according to claim 15 or 17, which grows in the condition of pH 9.8.

REMARKS

Favorable reconsideration is respectfully requested in view of the foregoing amendments and the following remarks.

With respect to Applicants' claim for foreign priority, Applicants note that the Examiner has failed to acknowledge Applicants' claim for foreign priority or acknowledge that all certified priority documents have been received. Applicants respectfully request such acknowledgment in the next Official Action.

Claim 1 has been cancelled without prejudice and rewritten as new claim 14. Further, claims 4, 6 and 9 have been amended to depend on new claim 14 and to put the claims in better form under U.S. practice. Also, new claims 15-22 have been added to further protect specific embodiments of the present invention. Support for the claim amendments and new claims is readily apparent from the teachings of the specification (in particular, the Examples) and the original claims. Specifically, new claims 15, 16 and 18-21 are supported by Examples 1 and 2, and new claims 15, 17 and 22 are supported by Example 3.

With regard to the rejection of claims 1, 4, 6, 9 and 10 under 35 USC § 112, second paragraph, Applicants believe that each ground of rejection has been overcome by the amendments to the claims. Specifically, the phrases "many more" and "derived from" have been omitted in new claim 14 (which replaced original claim 1). Further, the limitation "the second time" is now more clearly presented in new claim 14. Thus, in view of these amendments, Applicants believe that this rejection can no longer be sustained and should be withdrawn.

With regard to the rejections of claims 1, 9 and 10 under 35 USC § 112, first paragraph, these rejections are deemed to be untenable in view of the amended claims and are thus respectfully traversed. Since the claims are now directed using *a mutator gene which causes a defect in the mutation repair mechanism of a cell under a certain condition* which the Examiner has indicated to be enabling and which the Examples have demonstrated the inventors to be in possession of, Applicants respectfully request that these rejections be withdrawn.

With regard to the rejection of claims 1, 4, 6, 9 and 10 under 35 USC § 103(a) as being unpatentable over Fijalkowska et al. and Lin et al. in view of either Imamoto et al. or Iwaki et al. and further in view of Pan et al. (1996 Antimicrobial Agents and Chemotherapy 40:2321-2326), this rejection is deemed to be untenable and is thus respectfully traversed.

To establish a *prima facie* case of obviousness, the cited references in combination must teach or suggest the invention as a whole including all the limitations of the claims. Here, in this case, none of the cited references teach or suggest the method of the newly amended and added claims. Further, none of the cited references suggest the superior effects of the claimed invention which makes it possible to establish a mutant of *Escherichia coli* strain of new claims 18-22. For

example, although Pan et al. disclose an *E. coli* strain which grows in the presence of 100 μg/ml of ciproflaxacin, the claimed method succeeds in establishing a mutant strain tolerant to 500 μg/ml of ofloxacin. Please note that both ciproflaxacin and ofloxacin belong to the "new quinolone drug" and the cellular mechanism of those drugs' activity is the same.

Thus, Applicants believe that in light of the above, this rejection also cannot be sustained and should be withdrawn.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned **"Version with markings to show changes made."**

In view of the foregoing amendments and remarks, it is respectfully submitted that the Application is now in condition for allowance. Such action is thus respectfully solicited.

If, however, the Examiner has any suggestions for expediting allowance of the application or believes that direct communication with Applicants' attorney will advance the prosecution of this case, the Examiner is invited to contact the undersigned at the telephone number below.

Respectfully submitted,

Kiyoshi TANABE et al.

By: _____

Lee Cheng
Registration No. 40,949
Attorney for Applicants

LC/gtg
Washington, D.C. 20006-1021
Telephone (202) 721-8200
Facsimile (202) 721-8250
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VERSION WITH MARKINGS TO SHOW CHANGES MADE

The claims have been amended as follows.

4. (Twice Amended) The method ~~of mutation~~ according to claim ~~1~~14, wherein the mutator gene is one or more mutator genes selected from the group consisting of dnaQ, dnaE, mutL, mutS, mutH, uvrD and dam.

6. (Twice Amended) The method according to claim ~~1~~14, wherein the certain condition for causing the defect ~~of~~in the mutation repair mechanism is a certain temperature.

9. (Thrice Amended) A mutant of a cell, ~~wherein~~said mutant comprising a mutation ~~is introduced into the~~in its genomic DNA ~~of the cell~~introduced by any one of the methods ~~of~~according to claims ~~1~~14, 4 ~~and~~or 6.

Version with Markings to Show Changes Made

A METHOD OF MUTAGENESIS

1. Technical Field of the Invention

The invention of this application relates to an effective and highly efficient method for introducing a random mutation in which mutation can be efficiently introduced into a cell or an organism individual and also a risk of extinction of treated cell or individual groups can be reduced, and this invention also relates to mutants and mutated phenotypic gene obtained by the said method.

2. Description of the Related Art [Background Art]

With regard to an art for a genetic modification of cells or organism individuals, a method where mutagen such as ultraviolet ray, radioactive ray or mutagenic substance is applied to cells or organism individuals, a method where exogenous gene is introduced into cells or organism individuals to modify by means of genetic engineering, etc. are available. In the case of induction of mutation in specific gene, there has been known a method where genetic engineering means such as site-specific mutation induction and *in vitro* mutation induction by accumulation of replication mistake in DNA utilizing a PCR amplifying technique.

Generally, when the site into which gene or mutation to

mutation into gene on plasmid introduced into a host, it is not suitable for a genetic modification of the host itself.

As mentioned above, in the conventional method for the introduction of a random mutation into cells or organism individuals, introduction of many mutations and avoidance of extinction of mutation-introduced group are in a relation of antinomy whereby it is difficult to obtain various and useful mutants in an efficient manner.

The invention of this application has been achieved in view of the above circumstances and its object is to provide a method where a random mutation is introduced into a cell or an organism individual in a high mutation rate and, at the same time, risk of extinction of treated group is reduced and useful and various mutants are efficiently obtained.

Summary of the Disclosure of Invention

As an invention for solving the above-mentioned matters, this application provides a method for mutagenesis of a gene, which comprises introducing much more point mutations into one strand of double-stranded genomic DNA of cell or organism individual than into another strand.

The first preferred embodiment of the method of the

present invention is that the point mutation is randomly introduced into four bases constituting the double-stranded genomic DNA.

The second preferred embodiment of the method of the present invention is that the cell or the organism individual is a mutant cell strain or a mutant organism individual having mutator gene in a mutation repair mechanism gene group. Incidentally, such a mutant cell strain or a mutant organism individual may inherently have the mutator gene or may be that into which extrinsic mutator gene is introduced.

The third preferred embodiment is that, in the above-mentioned method, the mutator gene is one or more mutator gene(s) selected from a group consisting of dnaQ, dnaE, mutL, mutS, mutH, uvrD and dam.

The fourth preferred embodiment of the above-mentioned method is that the mutator gene is a gene which causes a defect of mutation repair mechanism under a certain condition.

The fifth preferred embodiment of the method is that the condition for the defect of the mutation repair function is a certain temperature.

The sixth preferred embodiment of the method is that a step of introduction of mutation into genomic DNA under a certain condition and a step of selection of mutant under a selected pressure condition without introduction of mutation are repeated.

The seventh preferred embodiment of the method is that the step of introduction of mutation at the second time and thereafter are carried out under the same selected pressure as that in the step of mutant selection immediately therebefore.

As another invention, this application also provides a mutant of cell or organism individual where mutation is introduced into genomic DNA by any of the above-mentioned methods, and also provides a mutated gene which is isolated from the said mutant.

Brief Description of Drawings

Fig. 1 is ampicillin concentration-survival curve of dnaQ49 strain before introduction of mutation and a wild type.

Fig. 2 is ampicillin concentration-survival curve showing ampicillin sensitivity of dnaQ49 strain after the first introduction of mutation, a wild type and an MNNG wild type.

Fig. 3 is ampicillin concentration-survival curve

showing ampicillin sensitivity of dnaQ49 strain after the second introduction of mutation, a wild type and an MNNG wild type.

Fig. 4 is ampicillin concentration-survival curve showing ampicillin sensitivity of dnaQ49 strain after the third introduction of mutation, a wild type and an MNNG wild type.

Fig. 5 is ampicillin concentration-survival curve showing ampicillin sensitivity of dnaQ49 strain after the fourth introduction of mutation, a wild type and an MNNG wild type.

Fig. 6 is ampicillin concentration-survival curve showing ampicillin sensitivity of dnaQ49 strain after the fifth introduction of mutation, a wild type and an MNNG wild type.

Fig. 7 is ampicillin concentration-survival curve showing ampicillin sensitivity of another dnaQ49 strain after the fifth introduction of mutation.

Fig. 8 is an agarose electrophoresis of DNA fragments derived from ampC genes from each of a wild type $dnaQ^+$ strain and a dnaQ49 strain.

Description of the Preferred Embodiments

~~Best Mode for Carrying Out the Invention~~

Natural mutation is fixed according to the following particulars. First, injury is generated by oxygen radicals or intravital metabolites which physically or chemically affect the chromosomal DNA or erroneous base pair is resulted

restriction enzyme TSPE I before the PCR took place whereby no PCR amplification was resulted.

On the other hand, lanes 4 and 5 are the result where the PCR products of DNA fragment derived from dnaQ49 strain ampC genes. As being obvious from the result shown in the lanes 4 and 5, no band showing the presence of a PCR product of 100 bp was observed in the DNA fragment (lane 5) hybridized with a probe for measuring the leading chain (i.e., no mutation took place in the leading chain) while, in the case of the DNA fragment (lane 4) hybridized with a probe for measuring the lagging chain, a band showing the presence of a PCR product of 100 bp was observed.

From the above result, it has been confirmed that, in the case of *Escherichia coli* dnaQ49 strain, much more mutations are accumulated in the lagging chain than in the leading chain even in the level of genomic DNA.

~~Industrial Applicability~~

In accordance with the invention of this application, it is now possible to efficiently and effectively construct various useful mutants of microorganisms, cells or organism individuals. It is also now possible by analyzing the mutating conditions of the gene to clarify the mechanism of drug resistance, to estimate the occurrence of a novel insensible microorganism or to develop a drug therefor, to analyze the

Claims

What is claimed is:

1. A method for mutagenesis of a gene, which comprises introducing much more point mutations into one strand of double-stranded genomic DNA of cell or organism individual than into another strand.

2. The method according to claim 1, wherein the point mutations are randomly introduced into four kinds of bases.

3. The method according to claim 1 or 2, wherein the cell or the organism individual is mutant cell strain or mutant organism individual having mutator gene in a mutation repair gene group.

4. The method mutation according to claim 3, wherein the mutator gene is one or more mutator genes selected from a group consisting of dnaQ, dnaE, mutL, mutS, mutH, uvrD and dam.

5. The method according to claim 3 or 4, wherein the mutator gene is a gene which causes a defect of mutation repair mechanism under a certain condition.

6. The method according to claim 5, wherein the condition for the defect of the mutation repair mechanism is a certain

Abstract of the Disclosure

This application provides a method for mutagenesis of a gene, which comprises introducing much more point mutations into one strand of double-stranded genomic DNA of cell or organism individual than into another strand. In accordance with such a method, it is now possible to efficiently and effectively construct various useful mutants of microorganisms, cells or organism individuals. It is also now possible by analyzing the mutating conditions of the gene to clarify the mechanism of drug resistance, to estimate the occurrence of a novel insensible microorganism or to develop a drug therefor, to analyze the mutation of an oncogene and the mechanisms of cancer metastasis and increase in malignancy, to develop a therapeutic method using these mechanisms, etc.